Award Number:
W81XWH-08-1-0631

TITLE:
Analysis of Dachsous2 in Breast Cancer Progression and Recurrence

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REPORT DATE: October 2011

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
1 October 2011	Final	Sept 2, 2008 - Sept 1, 2011

4. TITLE AND SUBTITLE

Analysis of Dachsous2 in Breast Cancer Progression and Recurrence

5a. CONTRACT NUMBER
W81XWH-08-1-0631

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Mt. Sinai Hospital,

Toronto, Ontario
Canada M5G 1X5

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, MD 21702

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Microarray analysis of tumors women with axillary node negative (ANN) breast cancer revealed that a cDNA for Dachsous 2 (Ds2) is one of the most highly discriminatory genes in tumors from patients who had experienced early recurrence, compared to tumors from patients who did not recur for at least ten years ($p= 1.1 \times 10^{-}$ by students t-test). This suggests that increased transcription of Ds2 may be a predictive indicator of recurrence in ANN breast cancer. Ds2 encodes a large cell adhesion molecule implicated in planar polarity and cell proliferation, To determine the significance of the increased levels of Ds2 in recurrence we will: 1) Validate gene expression array results and determine if Ds2 proteins levels increase in tumors 2) Determine if PCP or Hippo pathway gene expression is altered upon Ds2 overexpression. 3) Determine the effects of altering Ds2 levels on proliferation and tumor susceptibility in the mouse

15. SUBJECT TERMS
Breast Cancer, Ds2, planar cell polarity, Hippo pathway

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**		18	**19b. TELEPHONE NUMBER** *(include area code)*

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INTRODUCTION:
Microarray analysis of tumors women with axillary node negative (ANN) breast cancer revealed that a cDNA for *Dachsous 2* (*Ds2*) is one of the most highly discriminatory genes in tumors from patients who had experienced early recurrence, compared to tumors from patients who did not recur for at least ten years (p= 1.1 x 10-5 by students t-test). *This suggests that increased transcription of Ds2 may be a predictive indicator of recurrence in ANN breast cancer*. *Ds2* encodes a large cell adhesion molecule[1]. The *Drosophila* homolog of *Ds2*, *Ds,* functions as a planar cell polarity (PCP) ligand for the large cadherin Fat[2-5], and together Ds and Fat regulate tissue organization through a PCP signaling pathway. Recent data has also linked Ds with a newly described growth control pathway, the Hippo kinase pathway[6-9]. To determine the significance of the increased levels of *Ds2* in recurrence we set out to: 1) Validate gene expression array results and determine if Ds2 proteins levels increase in tumors 2) Determine if PCP or Hippo pathway gene expression is altered upon Ds2 overexpression. 3) Determine the effects of altering Ds2 levels on proliferation and tumor susceptibility in the mouse

BODY:
Task 1. ***Determine if Ds2 protein and transcript levels are increased in tumors from patients that have recurrent breast cancer***.
A) Generate anti-Ds2 antibody. Our first goal was to generate specific antibodies to Ds2 that could be used in paraffin section analysis of tumour samples from ANN patients. We initially generated 4 antisera to the entire cytoplasmic domain of Ds2, immunizing 2

Fig. 1

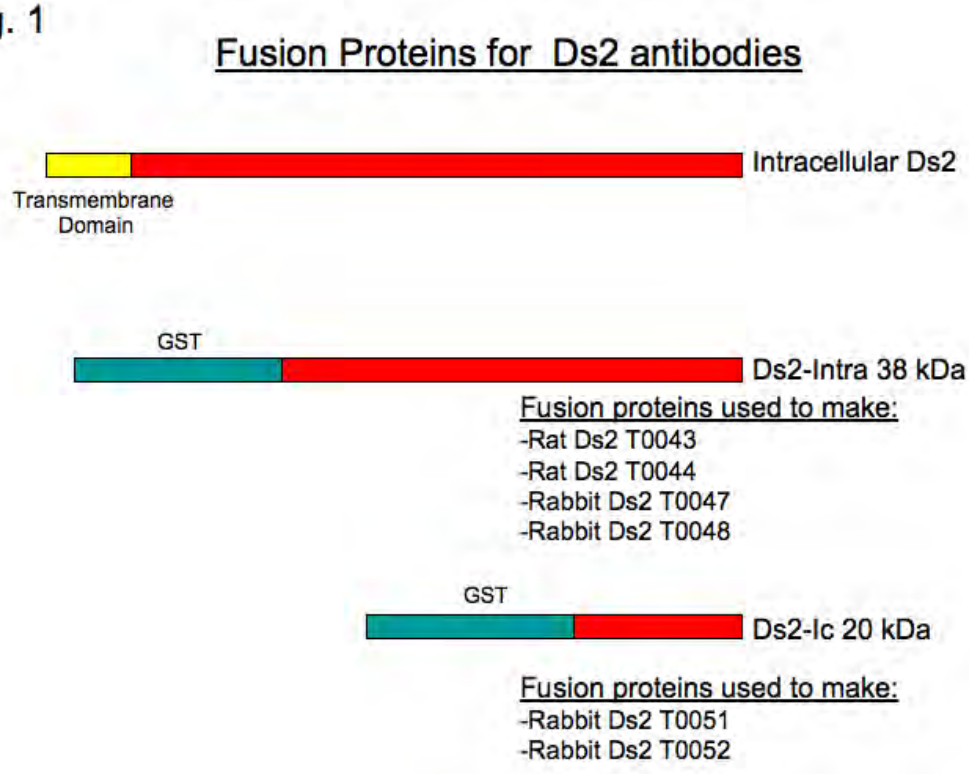


Figure 1. Constructs used to generate fusion proteins for specific antibodies to Ds2. Green indicates the GST portion, and red the segment of the intracellular domain of Ds2 used to generate antibodies.

rats and 2 rabbits (antibodies TO43, 44, 47 and 48: **Figure 1**). We chose to assay the antibodies using an immunohistochemistry of mouse hippocampus, as it has a simple and clear expression pattern of Ds2[1]. None of these antibodies showed any clear expression pattern in the hippocampus assay, despite trying a large number of staining conditions (we assayed cryosections and paraffin sections, tried a large dilution series, different blocking and different antigen retrieval approaches) (data not shown). We then generated fusion proteins to smaller portions of the cytoplasmic domain and inoculated 2 rabbits with this antigen (antibodies TO51 and TO52: **Figure 1**). These antibodies showed high specificity in both cryosection analysis and paraffin analysis of the hippocampus (**Figure 2**) and whole embryo staining (**Figure 3**).

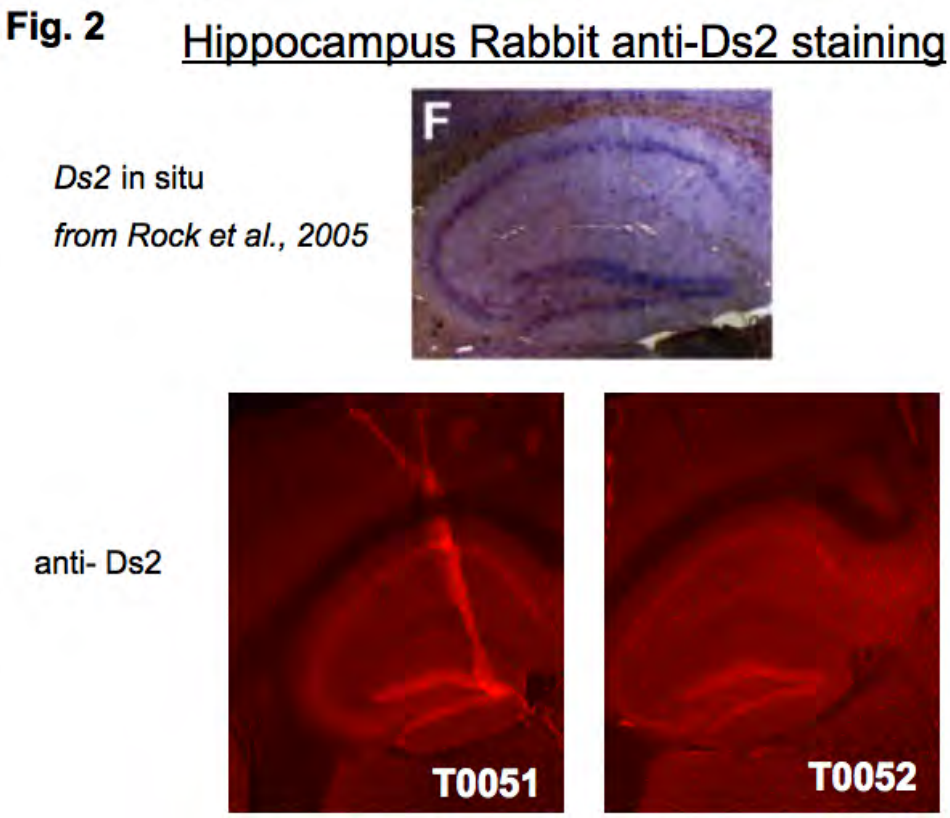


Figure 2. Validation of Ds2 antibodies. Top shows an in situ hybridization expression pattern of Ds2 in the hippocampus (taken from Rock et al., 2005). Bottom shows immunofluorescence of sections of the hippocampus, probes with antibodies to Ds2 (TO51 and TO52) that were effective in detecting Ds2 protein in immunofluorescence and western blot analysis.

Fig. 3 E12.5 embryo anti-Ds2 staining

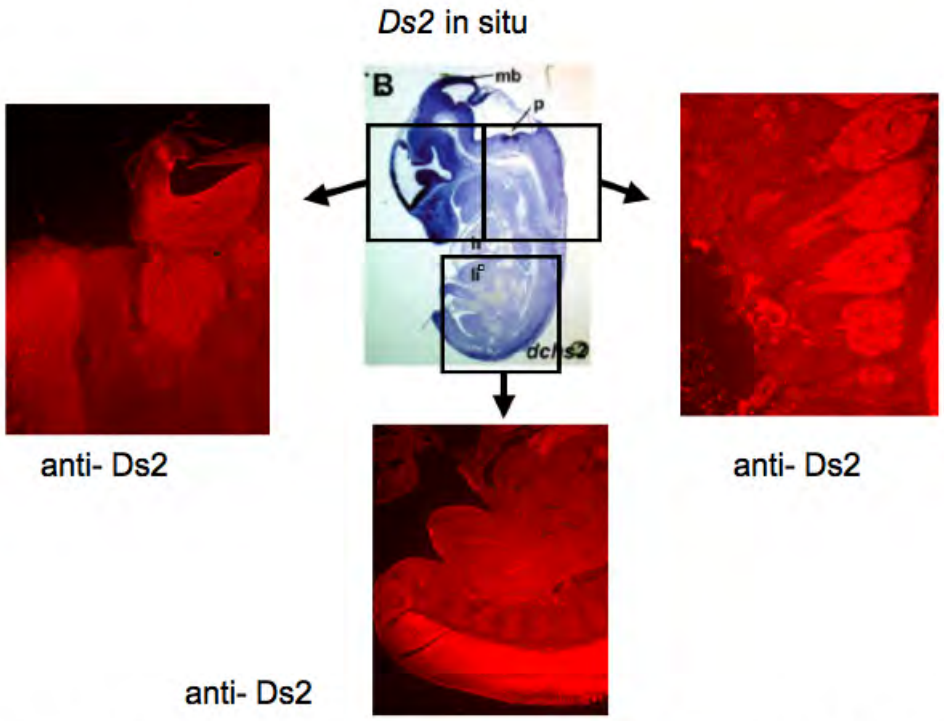


Figure 3. Immunofluorescence using specific Ds2 antibodies reveals the same pattern as published images of Ds2 by in situ hybridization (taken from Rock et al., 2005).

B) Test by RT-PCR if increased Ds2 expression validates in tumour samples. To determine if Ds2 mRNA is increased in tumour samples, we have used RT-PCR in collaboration with the laboratory of Dr. Irene Andrulis. Our initial studies assayed cell lines to optimize PCR conditions. Expression was normalized to the housekeeping gene, *hypoxanthine phosphoribosyltransferase 1* (HPRT1). We found that most cell lines express little or no Ds2 mRNA, although there was detectable expression in some cells such as SKOV3. The highest expression detected in cell lines was found in NTERA-2 cells (**Figure 4** and data not shown). Preliminary analysis of a panel of tumor samples revealed that most had low levels, on the order of most cell lines, however a few have very high levels, even higher than in NTERA-2 cells **(Figure 5**). This suggests that some recurrent tumors may indeed express unusually high levels of Ds2, however the finding thus far are not statistically significant, and more tumours must be assayed to reach a definitive conclusion.

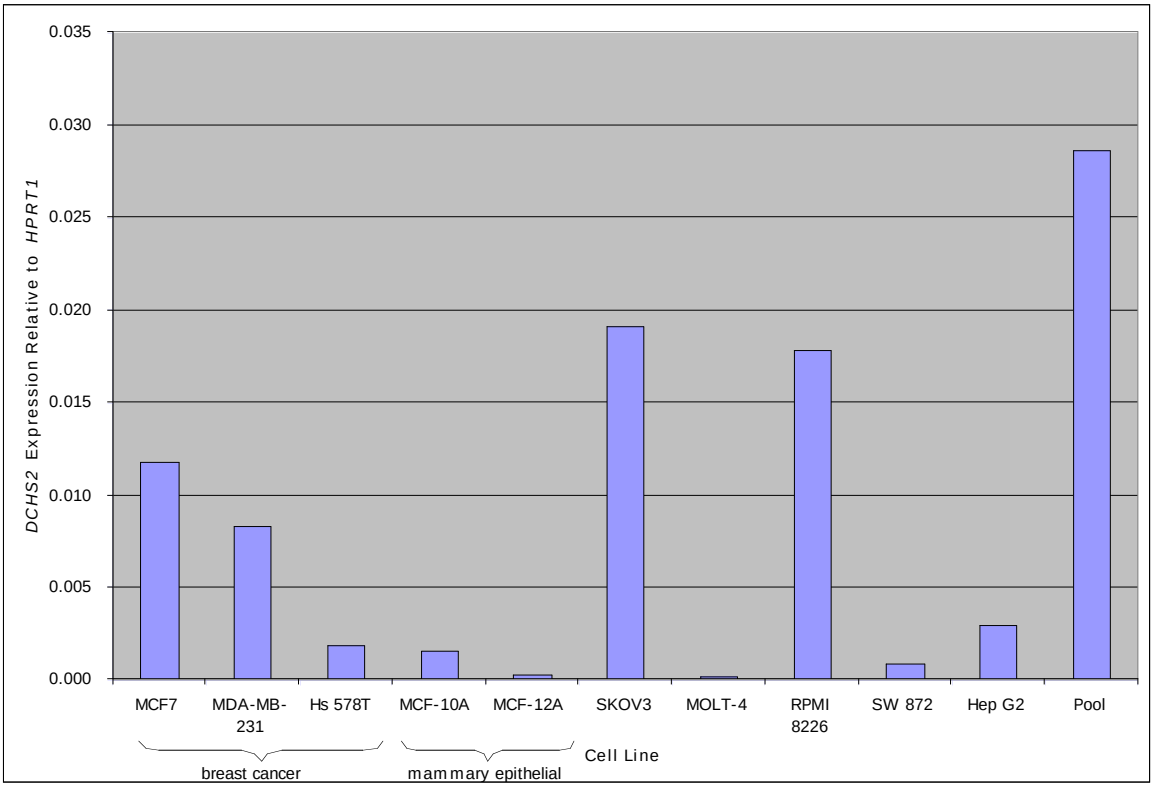


Figure 4. Quantifying expression of *dachsous 2* (DCHS2) in cell lines and a pool of 13 cell lines, using the real-time reverse-transcription polymerase chain reaction. Expression was normalized to the housekeeping gene HPRT1.

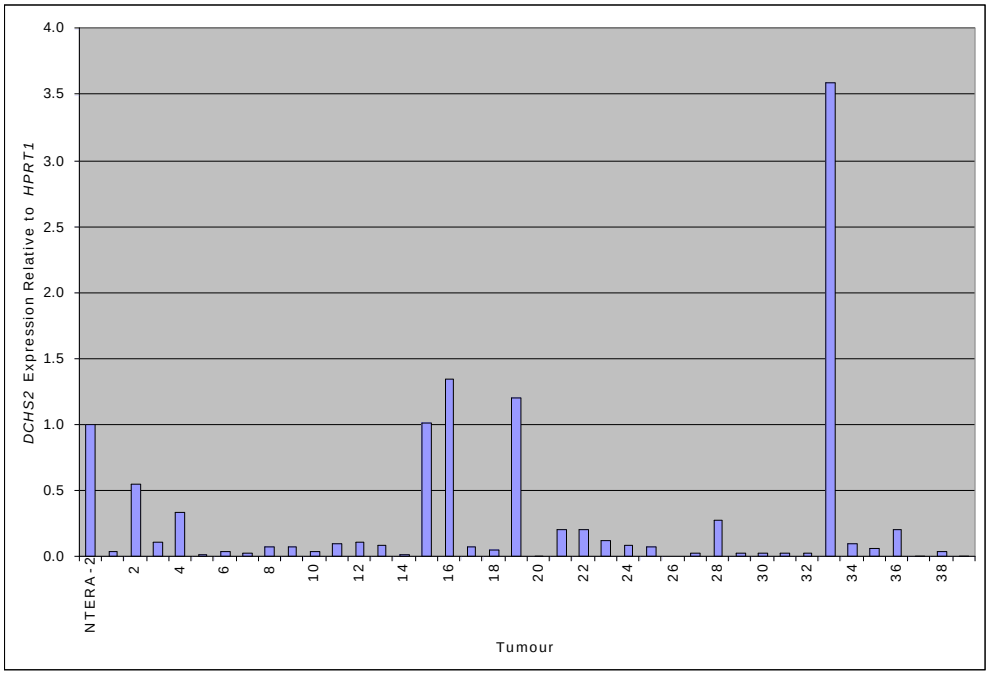


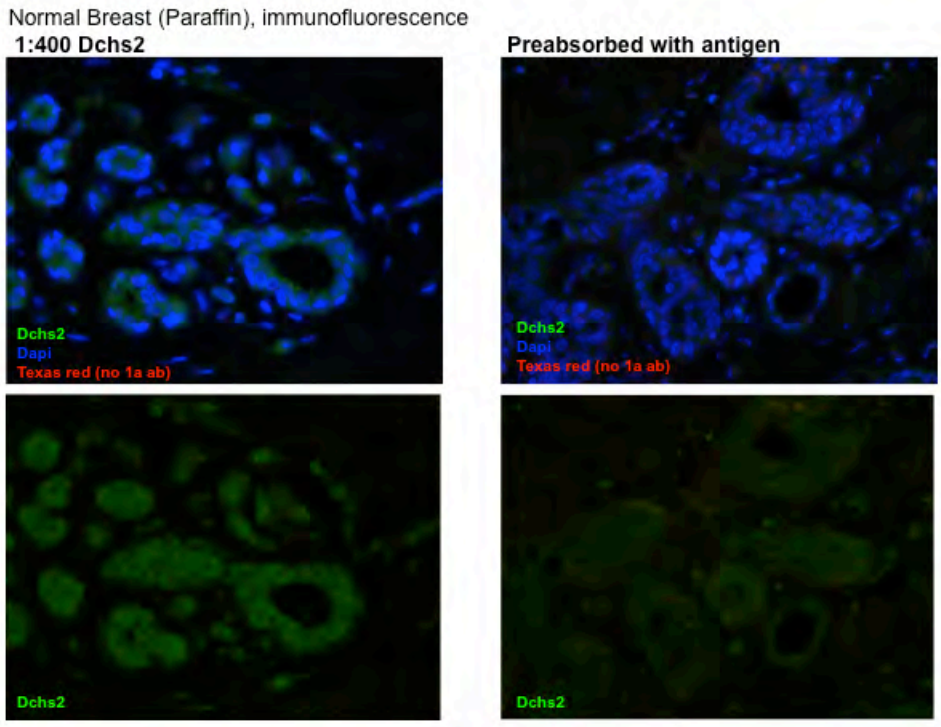
Figure 5. Analysis of Ds2 expression in tumours from ANN patients. Expression was low in most tumours but was very high in a subset of recurrent tumors.

Task 2. ***Determine if the Hippo or PCP pathway is activated with increased Dachous levels.***

1.A) Generate or obtain antibodies against Yap, MST1/2 and generate *in situ* probes for *fjx1. B)* Optimize staining on tissue sections. We have obtained antibodies to YAP and MST1/2 from commercial sources, and have generated *in situ* probes for *fjx1*. We optimized staining for these reagents using mouse embryos, however analysis of these probes on paraffin sections of human breast tissue indicated that these probes were not sufficiently specific to use in TMA analysis. We have successfully used the commercial YAP antibodies against mouse kidney samples and human breast tissue paraffin sections, and have validated its use in our TMA analysis- however analysis of the correlation between YAP localization and Dachsous expression must be delayed pending development of a suitable Ds2 antibody. Our extensive attempts to generate an antibody against Dachsous that is clean enough to use in TMAs have been thus far unscuccessful (see below).

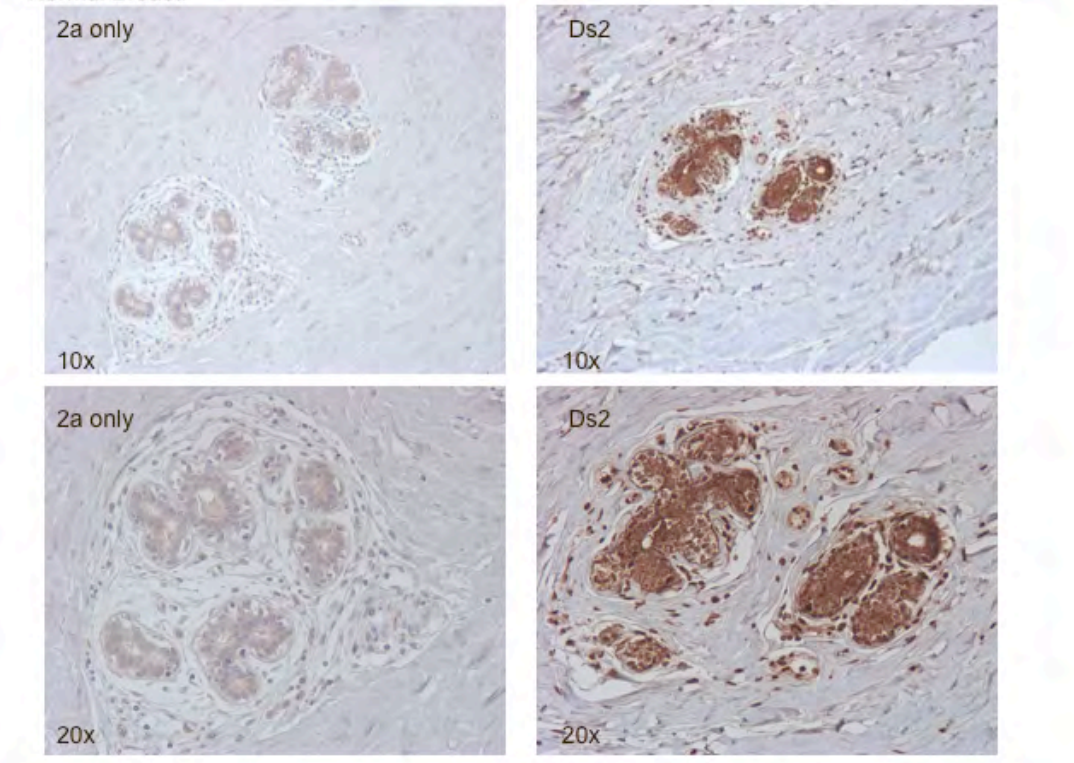
2A) Optimize TMA staining for Ds2. We have extensively now tested our Ds2 antibodies on normal breast samples and TMAs, with both positive and negative controls(**Figure 6 and Figure 7**). Preabsorbing with Dachsous antigen blocks staining, confirmed the specificitity of our antibodies on our preliminary TMA analysis, using immunofluorescence.

Figure 6. TMA Dchs2 Antibody Testing: Normal Breast



We found that Ds2 is expressed at cell-cell junctions in normal breast tissue, and find that in many breast tumours Ds2 becomes increased and expression and is often mislocalized. Our preliminary analysis of TMAs suggested there was high levels of Ds2 staining in many tumors, however upon repetition on a larger sample with DAB development, and upon consultation with a Pathologist (Dr. Ann Marie Weber), it was determined that the levels of background in these staining experiments was unacceptably high, and new antibodies must be generated. We also identified a recently developed commercially available antibody for Dachsous, however subsequent analysis of tumour samples using this antibody also resulted in unacceptably high background, precluding use in the TMAs

Figure 7. TMA Dchs2 Antibody Optimization with DAB



Task 3. ***Ascertain if overexpression of Ds2 affects tumour incidence or metastasis in mouse models of breast cancer.***

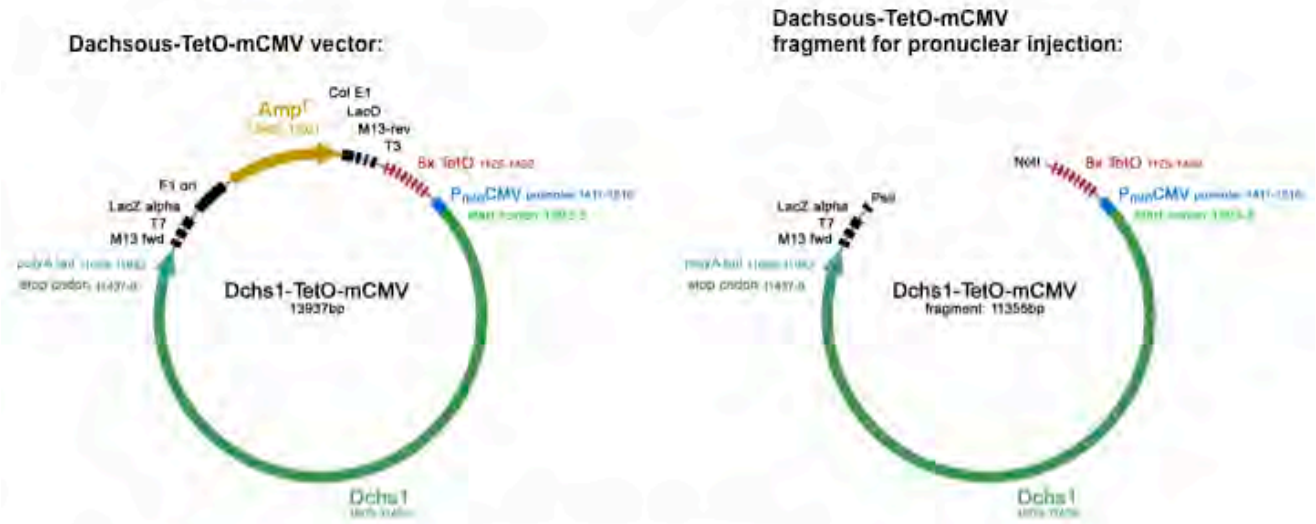
A) Generate construct for transgenic analysis of Ds2 function.

No functional studies have yet been conducted on Ds2 in mammals, and a full-length

Ds2 cDNA was not available. We initiated our cloning of Ds2 cDNA by conducting PCR from embryonic mouse cDNA libraries. We designed our PCR primers based on the available reference sequence predicted from the mouse genome. We generated embryonic cDNA libraries from E17.5 embryos. The full length Ds2 cDNA was predicted to be 8 kb, and we amplified the cDNA in several pieces, as we were unable to amplify the predicted full-length transcript. Sequencing of this transcript revealed several portions that were missing, in comparison to the predicted sequence. Examination of the mouse and human genome indicated that the missing sequences corresponded to predicted Ds2 exons. This indicated that there was unsuspected complexity in the splicing of this transcript.

In collaboration with Dr. Lisa Goodrich, we have isolated a number of independent Ds2 transcripts. This raises the question of which is the appropriate Ds2 cDNA to use for transgenic overexpression to assay the effects on the PCP and Hippo pathway in mice. All of the transcripts contain exon 25, which was on the microarray that detected increased *Ds2* in tumours from woman with recurrent breast cancer, therefore this cannot be used to discriminate among these alternate splice forms. To ensure that we used a functional splice form, tested these cDNA by transient transfection into cells that express Fat4. Functional Ds2 should be recruited to cell–cell junctions, upon interaction with Fat4. Generation of the predicted isoforms of Ds2 did not yield a functional protein. Examination of the extracellular domain of Ds1 and Ds2 isoforms indicated that Ds1 would function to activate the Hippo pathway as well as Ds2. A fully functional Ds1 cDNA was published by Dr. Tanoue's group in Japan, and shown to bind Fat4, the receptor for the pathway. We therefore obtained the cDNA from Dr. Tanoue, and cloned it into a tetracycline-inducible vector (**Figure 8)**).

Figure 8 Cloning for a Dachsous Tet-ON inducible transgenic mouse



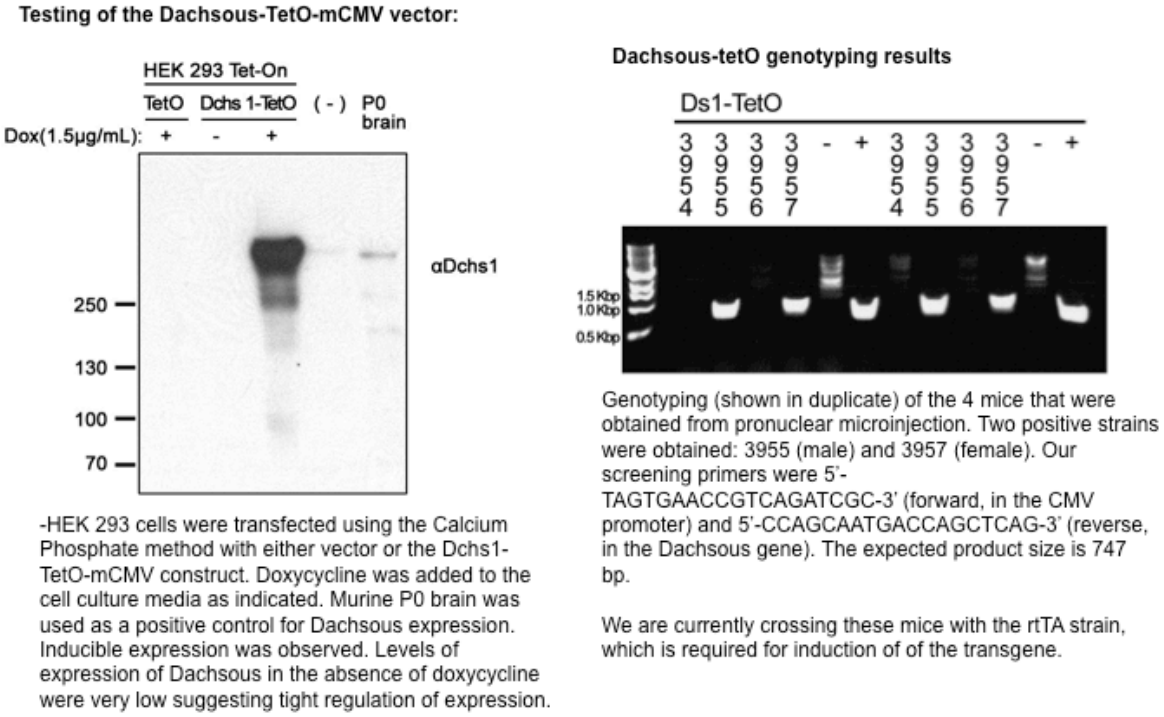
pA-TetO-mCMV vector:
-used for creation of a doxycycline inducible transgenic mouse with ubiquitous dachsous1 expression.

Cloning:
-The vector had a poly(A) tail located after the MCS. Dachsous (Dchs) cDNA was cloned into the MCS. The resultant TetO-dachsous-mCMV was digested with restriction enzymes and purified to remove bacterial sequences.
-The transgene was microinjected into pronuclei of the mouse zygotes (pronuclear microinjection) and implanted into pseudopregnant recipients. Pups were screened for the presence of a transgene at 2-3 weeks of age. Transgenic founders were identified by a PCR screening to identify mice that have the Dachsous-TetO sequence.
-embryos that were used were the result of the cross of C57BL/6 females with B6D2F1 (C57BL/6 x DBA/2) males.

To determine if the vector was suitable for inducible expression of Dachous, we tested with transient transfection in HEK293 cells that express the rtTA. These studies indicated that this vector conferred tight regulation (**Figure 9, left panel**).

To examine the *in vivo* effects of overexpression of Dachsous, we generated transgenic mice with this vector through pronuclear microinjection, and obtained two founder lines. Founder lines were confirmed to contain the Ds1-TetO trangene via PCR genotyping (**Figure 9, right panel**).

Figure 9. Generating a Dachsous Tet-ON inducible transgenic mouse



-HEK 293 cells were transfected using the Calcium Phosphate method with either vector or the Dchs1-TetO-mCMV construct. Doxycycline was added to the cell culture media as indicated. Murine P0 brain was used as a positive control for Dachsous expression. Inducible expression was observed. Levels of expression of Dachsous in the absence of doxycycline were very low suggesting tight regulation of expression.

Genotyping (shown in duplicate) of the 4 mice that were obtained from pronuclear microinjection. Two positive strains were obtained: 3955 (male) and 3957 (female). Our screening primers were 5'-TAGTGAACCGTCAGATCGC-3' (forward, in the CMV promoter) and 5'-CCAGCAATGACCAGCTCAG-3' (reverse, in the Dachsous gene). The expected product size is 747 bp.

We are currently crossing these mice with the rtTA strain, which is required for induction of of the transgene.

B) Determining the inducibility and bioactivity of a transgenic Dachsous 1 mouse line.

Having made transgenic mice, we then needed to determine if we could induce Dachsous expression in mice. We bred mice carrying Ds1-TetO with mice carrying rtTA, allowing us to induce expression in mice by feeding them doxycyline. To maximize our chances of detecting phenotypes in Planar Cell Polarity, we fed mothers starting at E0. Mice overexpressing Dachsous died at birth, with a curly tail (**Figure 10**). As a curly tail is a common phenotype of PCP genes, this suggested that the overexpression of Dachsous was competent to regulate external PCP phenotypes. As mice did not have altered overall size, this suggests that the regulation of growth, and by extension the Hippo pathway, was not affected by Dachsous overexpression.

Figure 10. Mice overexpressing Dachsous die at birth with curly tails.

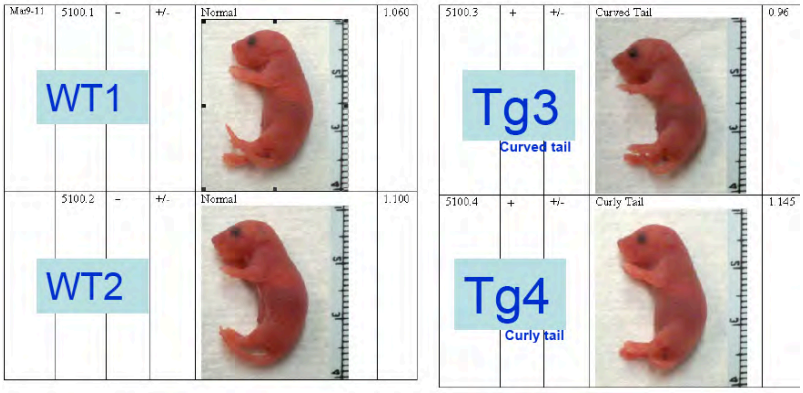
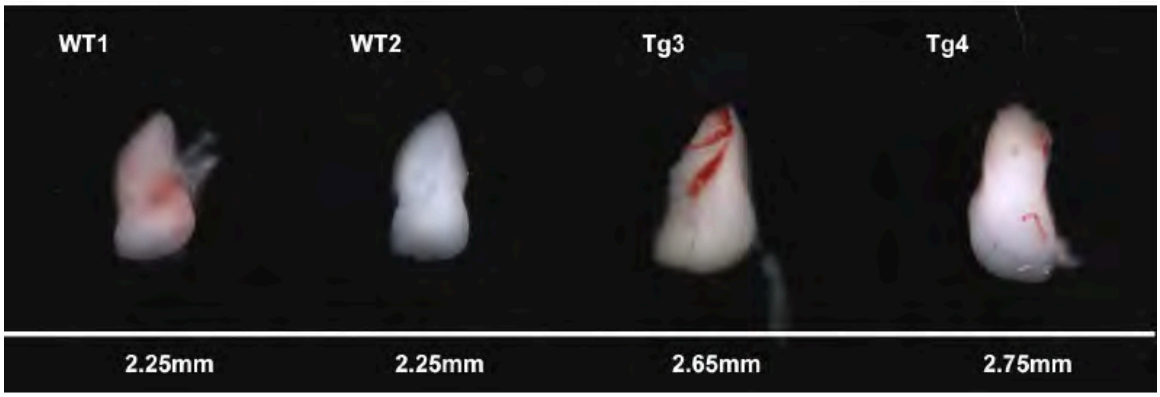


Fig. 10. Sibling mice at birth. Mice overexpressing Dachsous1 (Tg3 and Tg4) die at birth, with distinctive curly tails and overall normal body size.

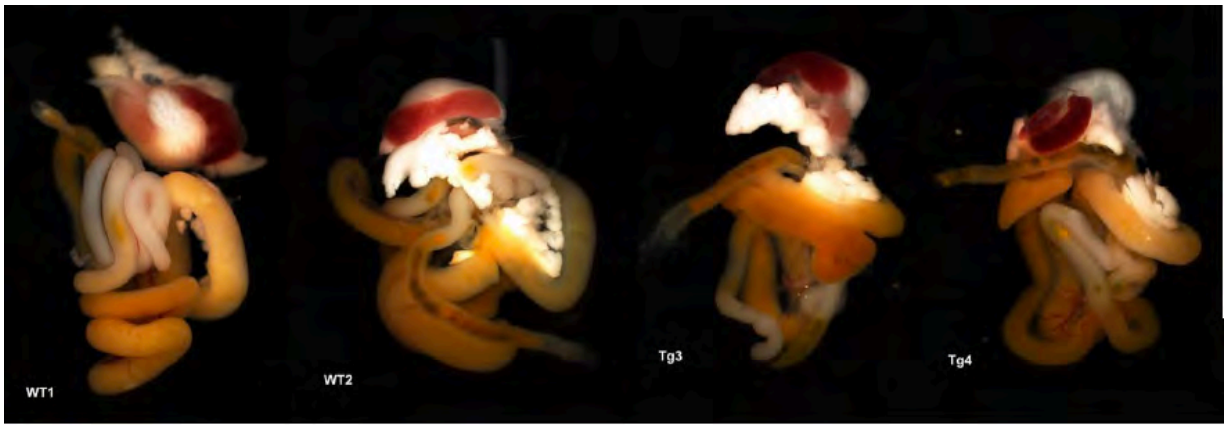
We then examined the internal organs of the Dachsous overexpressing mice. There was an increase in thymus size in mice that overexpressed Dachsous, compared to wildtype siblings (**Figure 11**).

Figure 11. Increased thymus size in mice overexpressing Dachsous



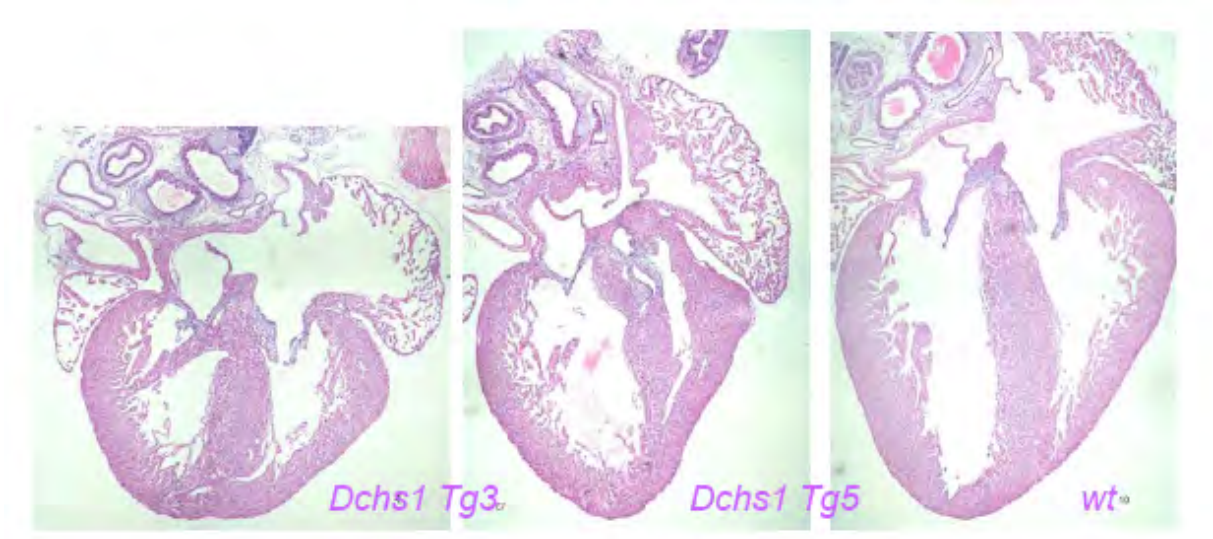
Studies with mice that are mutant for some PCP genes show shortening of the intestines. Despite a normal overall body size, Dachsous overexpressing mice show a shorter small intestine, consistent with a function in controlling planar polarity (**Figure 12**).

Figure 12. Shortened intestinal length in Dachsous1 overexpressing mice.



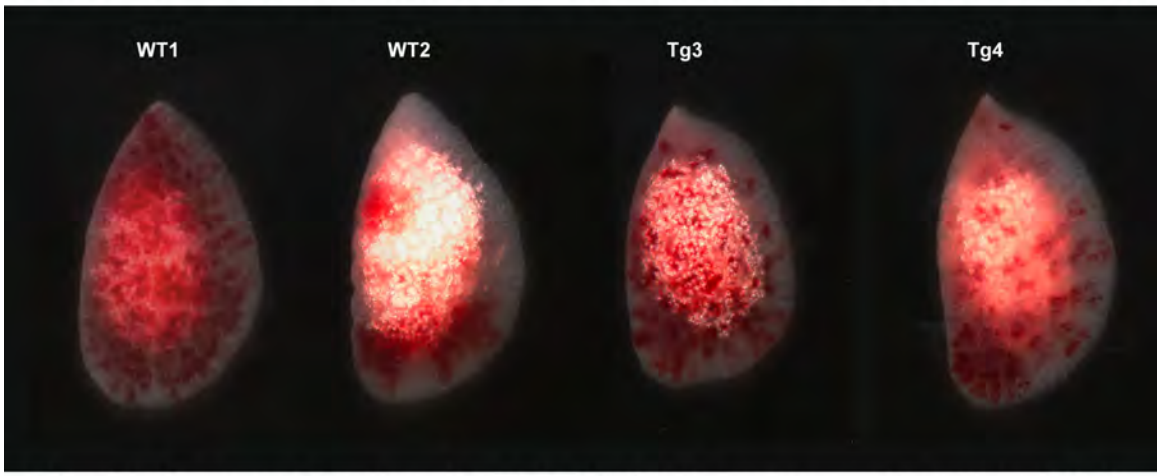
Recent studies have shown that loss of Dachsous 1 specifically affects heart formation. Despite a normal overall body size, the hearts of Dachsous1 overexpressing mice are small, with an abnormal shape and defective valves (**Figure 13**). This supports the idea that overexpressed Dachsous 1 functions in the same tissues in organizing heart morphology.

Figure 13. Overexpressed Dachsous leads to small heart size and defective heart morphology.



We also examined the effects of overexpressing Dachsous on liver and lung development, but there were no detectable alterations in the overt morphology of these organs (**Figure 14** and **Figure 15**). Future studies are needed to determine if there are more subtle alterations in the functioning of these tissues.

Figure 14.
Dachsous overexpression does not cause overt changes in lung morphology



Left lung lobes from representative wildtype siblings (WT1 and WT2) and Dachsous overexpressing pups (Tg3 and Tg4). No consistent change was noted in the overall morphology in animals that overexpress Dachsous.

Figure 15.
Dachsous overexpression does not cause overt changes in liver morphology

Livers from representative wildtype siblings (WT1 and WT2) and Dachsous overexpressing pups (Tg3 and Tg4). No consistent change was noted in the overall morphology in animals that overexpress Dachsous.

In conclusion, we have generated a mouse that overexpresses Dachsous in an inducible manner, and that it functions in regulation of planar cell polarity *in vivo*. The embryonic lethality obviously precludes the use of mice treated during embryonic development for studies in cancer progression. We are currently examining the effects of feeding newborn and adult mice doxycyline, in order to avoid these lethal effects. These mice will then be aged to determine if there is spontaneous tumour formation, and challenged with tumor-promoting factors to detemine if there is increased sensitivity to tumour formation. In the future, we will specifically focus on experiments to determine if metastasis is altered, as

our studies have indicated that tissue organization is most sensitive to altered Dachsous levels.

KEY RESEARCH ACCOMPLISHMENTS:

- Determined novel splice forms of Dachsous
- Generated antibodies for Dachsous1 and Dachsous2
- Identified high levels of Ds2 in a subset of tumor cell lines.
- Validated specificity of Dachsous antibodies in western blot analysis
- Validated specificity of Dachsous antibodies in mouse embryo staining
- Generated mice transgenic for Doxycline-inducible expression of Dachsous
- Demonstrated using western blot analysis that doxycyline was effective in inducing Dachous overexpression in transgenic mice.
- Demonstrated that overexpressed Dachsous2 is biologically active in vivo.
- Determinated that overexpression of Dachsous2 embryonically leads to neonatal lethality, accompanied by dysplastic kidneys, increased thymus size, and defective heart development.

REPORTABLE OUTCOMES

-Presentation of this study as a poster at the Era of Hope 2012 Meeting in Florida, USA. Entitled " ROLE OF THE CELL ADHESION MOLECULE DACHSOUS IN BREAST CANCER"
- Used preliminary data acquired on overexpression in transgenic mice of Dachsous1 to apply for funding for future studies on Dachsous1/2 from the Canadian Institute of Health Research (CIHR).

CONCLUSION:

In previous studies we identified increased levels of *Dachsous* mRNA as a predictor of recurrence in woman with axillary node negative breast cancer. This lead to the hypothesis that increased levels of *Dachsous* mRNA and protein may provide a biomarker for recurrent breast cancer. To test this hypothesis, we have generated antibodies to Dachsous, cloned full length Dachous cDNAs and obtained antibodies and in situ probes to components of the mammalian Hippo and Planar Cell Polarity Pathway . We have also generated mice that can overexpress Dachsous upon treatment with Doxycline. We have shown that the overexpression of Dachsous1 does not affect the overall growth of embryos, but does alter their PCP. This suggests that the PCP functions of Dachsous, and not the proliferative functions are what is key in woman who overexpress Dachsous. This implies that it is defective PCP that leads to altered tissue organization and increased metastasis. We can use these tools to determine the signal transduction pathways that are disrupted when Dachsous is overexpressed.

APPENDICES:

1. Abstract of poster P6-12, Presented at Era of Hope Meeting 2012
ROLE OF THE CELL ADHESION MOLECULE DACHSOUS IN BREAST CANCER

Poster P6-12

BC073798-2635

ROLE OF THE CELL ADHESION MOLECULE DACHSOUS IN BREAST CANCER

Helen McNeill
Mount Sinai Hospital, Samuel Lunenfeld Research Institute

Ds2 encodes a large-cell-adhesion molecule with 27 cadherin repeats and a novel but highly conserved cytoplasmic domain. No studies have yet addressed the function of Ds1 or Ds2 in vertebrates. However, data from our lab and others have linked the Drosophila homolog of Ds2, Ds, with a tissue organization pathway, known as the planar polarity pathway (PCP). Ds functions as a PCP ligand for another large cadherin Fat, and together Ds and Fat regulate tissue organization through a PCP signaling pathway. Loss of PCP signaling has been implicated in a number of cancers. In addition, loss of Ds has been shown to lead to excess tissue growth in Drosophila. Recent data from our laboratory and others have also linked Fat and Ds with a newly described growth control pathway, the Hippo kinase pathway. Recent data have shown that the Hippo pathway is conserved to man and is misregulated in a variety of cancers, including breast cancers. Together, these findings suggest that altered levels of Ds2 in tumors from ANN patients that show recurrence of breast cancer may be linked to a misregulation of either the PCP pathway or the Hippo kinase pathway**.** Alterations in Ds2 PCP signaling may alter tissue organization in patients, leading to metastasis. Alternatively, disruptions of Ds2-mediated growth control might increase proliferation, predisposing cells to cancerous growth.

To determine the significance of the altered levels of Ds2 in recurrence, we will:

1. Determine if Ds2 proteins levels alter in tumors.

2. Determine if PCP or Hippo pathway gene expression is altered upon changing Ds levels.

3. Determine the effects of altering Ds2 levels on proliferation and tumor susceptibility in mouse models.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-08-1-0631.

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